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Y-shaped biotin-conjugated poly (ethylene glycol)–poly (epsilon-caprolactone) copolymer for the targeted delivery of curcumin

Wenxia Zhu [a,b], Zhimei Song [c], Peng Wei [d], Ning Meng [c], Fangfang Teng [a,b], Fengying Yang [c], Na Liu [c], Runliang Feng [c,*]

[a] School of Medicine and Life Sciences, University of Jinan-Shandong Academy of Medical Sciences, No. 16866 East Road of Jingshi, Jinan 250200, Shandong Province, PR China
[b] Shandong Academy of Medical Sciences, No. 18877 Jingshi Road, Jinan 250062, Shandong Province, PR China
[c] Department of Pharmaceutical Engineering, School of Biological Science and Technology, University of Jinan, No. 336 West Road of Nanxinzhuang, Jinan 250022, Shandong Province, PR China
[d] National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, No. 15 Datun Road, Chaoyang District, Beijing 100101, PR China

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ABSTRACT

In order to improve curcumin's low water-solubility and selective delivery to cancer, we reported ligand-mediated micelles based on a Y-shaped biotin-poly (ethylene glycol)–poly (epsilon-caprolactone)$_2$ (biotin-PEG–PCL$_2$) copolymer. Its structure was characterized by ^{1}H NMR. The blank and drug-loaded micelles obtained by way of thin-film hydration were characterized by dynamic light scattering, X-ray diffraction, infrared spectroscopy and hemolytic test. Curcumin was loaded into micelles with a high encapsulating efficiency (93.83%). Curcumin's water-solubility was enhanced 170,400 times higher than free curcumin. Biotin-PEG–PCL$_2$ micelles showed slower drug release *in vitro* than H_2N–PEG–PCL$_2$ micelles. *In vitro* cellular uptake and cytotoxicity tests showed that higher dosage of curcumin might overcome the effect of slow release on cytotoxicities because of its higher uptake induced by biotin, resulting in higher anticancer activities against MDA-MB-436 cells. In brief, Y-shaped biotin-PEG–PCL$_2$ is a promising delivery carrier for anticancer drug.

1. Introduction

Curcumin, a hydrophobic polyphenol derived from the rhizome of the herb Curcuma longa, has shown health promoting and disease preventing activity against several diseases including cancer [1]. However, several studies have revealed extremely low water-solubility, unstable property, rapid metabolism and poor absorption of this molecule that severely reduces its bioavailability [2,3]. To overcome these problems, drug delivery systems (DDS) have been taken into consideration to provide longer circulation times, increased permeability, and resistance toward metabolic presystemic degradation [4,5]. It is well known that the main problem of cancer therapy is how to deliver anticancer drug to tumor organ or tissue to improve its therapeutic efficiency and decrease its side-effect. Active target technology has been intensively researched for solving it with significant development [6–10]. In this area, some small molecular target compounds, such as folic acid [11], galactose [12], arginine-glycine-aspartic acid (Arg-Gly-Asp, RGD) [13] and forth, have been used to decorate polymeric particle for enhancing curcumin's active targeting property. It is noted that biotin is used widely as a tumor targeting ligand for various anti-cancer drugs [14–20]. Overexpression of its receptor on the surface of different cancer cells can introduce the biotin-conjugated copolymeric drug delivery system into them [20]. In our previous research, a Y-shaped methoxy poly (ethylene glycol)–b-poly (epsilon-caprolactone) (MPEG–PCL$_2$) block copolymer can provide a higher encapsulation efficiency for curcumin with sustained release property [21]. So, it is believed that drug delivery system based on Y-shaped biotin-conjugated poly (ethylene glycol)–poly (epsilon-caprolactone) (biotin-PEG–PCL$_2$) copolymeric micelles cannot only afford better encapsulation and release property for curcumin, but also realize selective delivery of it to cancer cells.

In this study, the biotin-PEG–PCL$_2$ copolymer was synthesized through O-alkylation, alkaline ring-opening, radical addition, ring-opening polymerization and acylation reaction with monoallyloxy poly (ethylene glycol) ether as raw material. The structure of the copolymer was confirmed by ^{1}H NMR spectroscopy. The micelles were prepared by thin-film hydration with a higher drug loading capacity. We also characterized the micelles in the terms of dynamic light scattering, X-ray diffraction, infrared spectroscopy

* Corresponding author. Fax: +86 531 89736818.
E-mail address: runliangfeng@126.com (R. Feng).

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and hemolytic test. The *in vitro* cytotoxicity against MDA-MB-436 cells was evaluated.

2. Materials and experiments

2.1. Materials

Curcumin was purchased from Fluka Chemical Company Inc. (Buchs, Switzerland). Monoallyloxy poly (ethylene glycol) (APEG, Mn = 2400) was procured from Haian petrochemical factory (Haian, Jiangsu Province, China). epsilon-Caprolactone (ε-CL) was provided by Huayuan polymer Co., Ltd. (Qingdao, Shandong Province, China). Epichlorohydrin purchased from Tianjin Damao Chemical Corp. (Tianjin, China) was used without further purification. Azodiisobutyronitrile (AIBN) was purchased from Fuyu Fine Chemical Co., Ltd. (Tianjin, China). N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and N-hydroxy succimide (NHS) were bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Biotin was purchased from Hongxiang Chemical Engineering Co., Ltd. (Zhengzhou, China). Methylene dichloride was dried under calcium hydride and distilled before use. All the other chemicals and solvents were of analytical grade or higher, obtained commercially.

2.2. Synthesis of biotin-PEG–PCL_2

2.2.1. Allyl-PEG–OH_2

Allyl-PEG–OH_2 was prepared according to a procedure about dihydroxy MPEG described previously with a modification [22]. Sodium (2.3 g, 100 mmol) was cut into pieces and added into a mixture of APEG (60 g, 25 mmol) and tetrahydrofuran (200 mL). The mixture was agitated at 60 °C for 8 h under nitrogen flow. Then epichlorohydrin (13.93 g, 150 mmol) was added dropwise and reacted at the same temperature for another 12 h. The solvent and excessive epichlorohydrin were removed by evaporation under vacuum after filtration at room temperature to afford allyl-PEG glycidyl ether. This ether was mixed with 100 mL of 20% sodium hydroxide solution and heated at 80 °C for 24 h. The mixture was evaporated under vacuum to dry. The residual was redissolved in methylene dichloride and dried with anhydrous sodium sulfate overnight. The most of solvent was removed under vacuum and precipitated in diethyl ether to afford white powder solid as product.

2.2.2. H_2N–PEG–$(OH)_2$

H_2N–PEG–$(OH)_2$ was prepared in accordance with a synthetic process for H_2N–PEG–OH reported in a previous article [23]. Briefly, Ally-PEG–OH_2, cystamine hydrochloride, and AIBN were co-dissolved and stirred in anhydrous N, N-dimethyl formamide (DMF) at 65 °C for 24 h under nitrogen atmosphere. The solvent was evaporated to dry under vacuum. Potassium carbonate solution was added into the residual to remove excess cystamine hydrochloride and distilled to dry. To the residual methylene chloride was added, mixed well and filtrated in turn. After evaporation of the majority of filtrate, diethyl ether was added to afford amino-PEG–OH_2 as sediment.

2.2.3. Biotin-PEG–$(OH)_2$

Biotin was treated with NHS and EDC in anhydrous DMF at room temperature for 24 h [24]. The resulting mixture was added into anhydrous methylene chloride containing H_2N–PEG–$(OH)_2$ and stirred at room temperature for 24 h. The solvent was evaporated. The residual was recrystallized from isobutyl alcohol to afford the target product [25].

2.2.4. Biotin-PEG–PCL_2

Biotin-PEG–PCL_2 was synthesized according to a preparation method reported previously [25,26]. To remove the last traces of water, the biotin-PEG–$(OH)_2$ was azeotroped with toluene at 120 °C for 4 h. The toluene was removed by evaporation. To the residual methylene dichloride, hydrochloride diethyl ether and different weight of ε-CL were added in turn. The reaction mixture was stirred at room temperature for 24 h. The resultant mixture was evaporated under vacuum and precipitated in diethyl ether to obtain the biotin-PEG–PCL_2 copolymer after filtration. The obtained precipitate was vacuum-dried to constant weight and stored in a desiccator before use.

^{1}H NMR spectra of allyl-PEG–OH_2, H_2N–PEG–$(OH)_2$ and biotin-PEG–$(OH)_2$ in DMSO-d_6 were recorded with a 400M NMR Bruker AVANCE spectrometer (Bruker BioSpin, Germany), and ^{1}H NMR spectra of biotin-PEG–PCL_2 in $CDCl_3$ were recorded with a 400 M NMR Bruker AVANCE spectrometer (Bruker BioSpin, Germany).

2.3. Preparation of blank and curcumin-loaded micelles

Micelles were prepared by thin-film hydration [27,28]. The known weight of curcumin and biotin-PEG–PCL_2 were co-dissolved in acetone, which was evaporated under vacuum at room temperature to obtain a thin layer of uniform film. The resulting yellowish thin film was hydrated under moderate stirring at 65 °C. The resulting solution was filtered through a 0.22 μm filter membrane to remove curcumin or some other substances undissolved and used for further analysis or lyophilization. Blank micelles would be formed in a similar manner.

2.4. Characterization of micelles

The critical micelle concentration (CMC) of copolymer was determined using a fluorescent spectrophotometer with pyrene as the probe. Equivalent volume of pyrene solution in acetone (6×10^{-6} mol/L) was added into a series of tubes and evaporated under a nitrogen gas stream. Copolymer solution (10 mL), ranging from 1×10^{-3} to 5×10^{-8} g/mL, was added to each tube to achieve a final pyrene concentration of 6×10^{-7} mol/L. The fluorescence spectrum was measured using a fluorescent spectrometer.

To evaluate the safety of the copolymer for intervenous injection, hemolysis test was performed [29]. Briefly, centrifugation at 3000 r/min for 20 min was performed to separate heparinized rat erythrocytes from rat blood. The obtained erythrocytes were washed with physiological saline to achromaticity for supernatant. The purified erythrocytes were mixed with normal saline to afford a suspension of 2.5% (V/V), which was added into 4 mL of copolymer solution in normal saline at 37 °C to form a suspension of 1% (V/V) for erythrocytes. The mixture was shaken in an oscillator (300 r/min, 37 °C) for 30 min. The copolymer solution concentrations were 78, 156, 312, 625, 1250, 2500, and 5000 μg/mL, respectively. Negative (0%) and positive (100%) control were normal saline and distilled water, respectively. The samples were centrifuged for 20 min at 3000 r/min to remove unbroken erythrocytes and disrupted membranes. The ultraviolet spectrogram of released hemoglobin in the supernatant from 500 to 650 nm was recorded at a UV–Vis spectrophotometer (T6 New Century, Purkinje General, Peking, China), and the hemolytic percentage of the copolymer was calculated from equation as follow according to peak absorbance at 576 nm [30].

$$\text{Hemolysis}\ (\%) = \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{negative control}}}{\text{Abs}_{\text{positive control}} - \text{Abs}_{\text{negative control}}} \times 100\%$$

A known volume of curcumin-loaded micelle solution was mixed with ethanol in volumetric flask to dissolve curcumin. Curcumin's amount in micelles was analyzed by UV–Vis

spectrophotometer at 425 nm (T6 New Century, Purkinje General, Peking, China). The drug loading (DL) and entrapment efficiency (EE) were calculated based on the following formula [31].

$$EE = \frac{\text{weight of drug in micelles}}{\text{weight of the initial drug}} \times 100\%$$

$$DL = \frac{\text{weight of drug in micelles}}{\text{weight of micelles containing drug}} \times 100\%$$

The particle size and zeta potential of curcumin-free and loaded micelles were measured by dynamic light scattering (DLS, Zetasizer ZS90, Malvern Instruments Ltd., UK). Transmission electron microscope (TEM, JEM-1200EX, JEOL, Tokyo, Japan) was used for shape and morphology observation.

The X-ray diffraction analysis of curcumin, lyophilized curcumin-free and loaded micellar particles were performed by X-ray diffractometer (Bruker D8 Focus, Bruker, Germany) using Cu Kα radiation (40 kV, 30 mA).

FT-IR spectrum of curcumin, lyophilized curcumin-free and loaded micellar particles were afforded by KBr pellets on a FT-IR spectrometer (Spectrum One, PerkinElmer, USA) in a range from 3900 to 400 cm^{-1}, respectively.

2.5. In vitro release of curcumin-loaded micelles

In vitro drug release of curcumin from curcumin-loaded micelles was processed by way of dialysis method reported previously [21,23,32]. Curcumin-loaded micelles solution and control solution in ethanol containing 1 mg of curcumin were added into dialysis bag (Viskas MD25-3.5, Union Carbide Corporation, Bound Brook, NJ, USA) and put into 100 mL of release medium composed of ethanol and physiological saline (40:60 (V/V)) fitting for sink condition, respectively. Then they were continuously shaken in an oscillator (100 r/min, 37 °C). At definite time intervals, 5 mL of release medium was withdrawn, and the same volume of fresh release medium was added into the release medium. The amount of curcumin's release was determined by UV–Vis spectrophotometer at 425 nm (T6 New Century, Purkinje General, Peking, China). The experiment was performed in triplicates. The results were expressed as mean ± standard deviation (SD).

2.6. In vitro MTT cytotoxicity test

MDA-MB-436 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). DMEM containing 10% FBS was used as incubation medium of cells growth in a 5% CO_2 atmosphere at 37 °C [33]. Cytotoxicities of free curcumin, blank micelles and curcumin-loaded micelles against MDA-MB-436 cells were determined according to a MTT assay. MDA-MB-436 cells were sown with a density of 5×10^3 per well and incubated overnight. After eliminating incubation medium, 100 μL of free curcumin, blank or curcumin-loaded biotin-PEG–PCL_2 and H_2N–PEG–PCL_2 micelles (0–40 μM) in incubation medium were added and continuously incubated for 24 or 48 h at 37 °C under the same condition, respectively. 100 μL of MTT reagent solution (5 mg/mL) was transported into the wells and incubated at 37 °C for 2 h prior to absorption of the solution, respectively. 100 μL of dimethyl sulfoxide was added to the wells to dissolve formazan formed during the incubation. The absorbance of formazan was measured at a wavelength of 490 nm in a microplate reader (Model 680; Bio-Rad Laboratories, Hercules, CA), and cell viability was determined. The experiments were performed in triplicates.

2.7. In vitro cell uptake

MDA-MB-436 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). DMEM containing 10% FBS was used as incubation medium of cells growth in a 5% CO_2 atmosphere at 37 °C [33]. After washed with PBS three times, the same volumes of cell suspensions were incubated with free curcumin, curcumin-loaded micelles at a curcumin concentration of 40 μM for 2 h at 37 °C. Ahead of being photographed, the cells were washed with PBS in triplicates in order to detect the intracellular curcumin merely. The excitation and emission wavelengths were 405 nm and 470–600 nm to observe cellular uptake of curcumin in free curcumin, biotin-PEG–PCL_2 and H_2N–PEG–PCL_2 micelles by confocal laser scanning microscopy, respectively. The pictures were analyzed by ImageJ 1.48V.

3. Result and discussion

3.1. Structure characterization of biotin-PEG–PCL_2

Biotin-PEG–PCL_2 was prepared according to the synthetic routine in Fig. 1. The allyl-PEG–OH_2 was obtained through

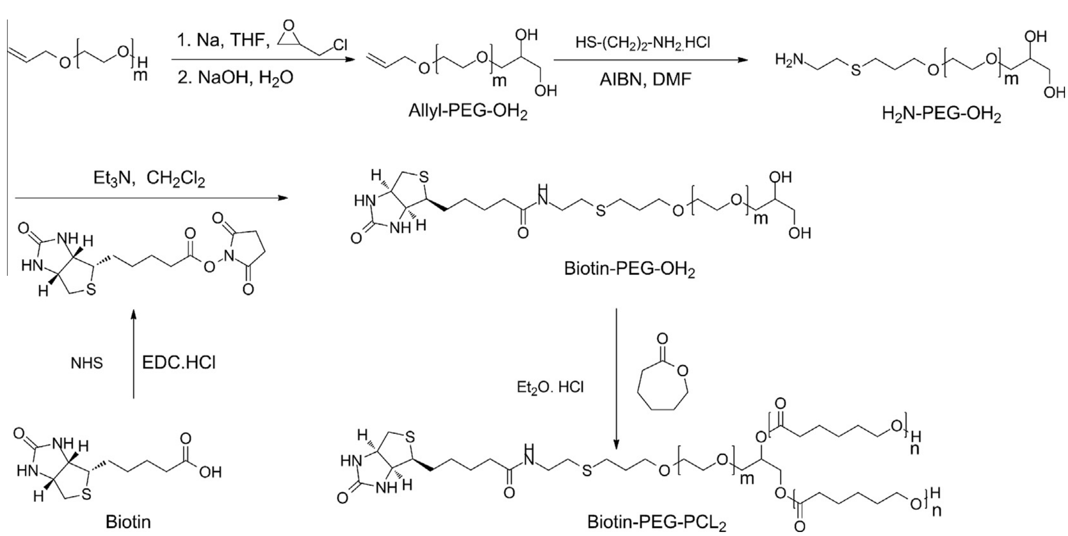

Fig. 1. Synthesis of biotin-PEG–PCL_2.

O-alkylation of APEG followed by hydrolytic ring-opening. Then radical addition reaction of allyl-PEG–OH_2 with cystamine hydrochloride afforded H_2N–PEG–OH_2, which went on with selective N-acylation of biotin under the treatment of NHS and EDC at room temperature to obtain biotin-PEG–OH_2. At last, biotin-PEG–PCL_2 was synthesized by way of ring-opening polymerization reaction of biotin-PEG–OH_2 with ε-CL under the condition of diethyl ether hydrochloride as catalyst.

The relevant ^{1}H NMR spectrum is listed in Supplementary Information (S 1). In the case of allyl-PEG–OH_2, peaks at 5.12–5.26 and 5.82–5.92 (S 1A) were characteristic signals of allyl group [34] that disappeared in the spectrum of NH_2–PEG–OH_2 (S 1B). At the same time, signals at 3.50, 3.34–3.42 and 3.68 shown in S 1A were assigned to —OCH_2CH_2O—, HOCH_2—CHOH—CH_2—O, and HOCH_2—CHOH—CH_2—O [35], respectively, which were similar to that in NH_2–PEG–OH_2. Peaks at 3.94 and 4.59 in double and triple showed the presence of two hydroxyl groups in allyl-PEG–OH_2, respectively [21]. Furthermore, a series of new peaks appeared at δ 1.72–1.74, 2.64 and 2.94 in the ^{1}H NMR spectrum of NH_2–PEG–OH_2, which were attribute to S—$CH_2CH_2CH_2$—O, S—$CH_2CH_2CH_2$—O and $NH_2CH_2CH_2$—S—$CH_2CH_2CH_2CH_2$—O, respectively [34,36].

As for biotin-PEG–OH_2 (S 1C), double peaks at 6.44 and 6.38 were characteristic hydrogen peaks of both biotin's and formed amides. Peaks at 4.59 and 4.29–4.14 indicated that there were two hydroxyl group [21], meaning that the N-acylation was selective with no formation of ester at the terminal hydroxyl of H_2N–PEG–OH_2. After ring-opening polymerization reaction, the hydroxyl group peaks disappeared (S 1D), and new peaks appearing at 1.36–1.42, 1.49–1.65, 2.29–2.37, and 4.05–4.08 ppm were assigned to the methylene protons of OOC—$CH_2CH_2CH_2CH_2CH_2$O, OOC—$CH_2CH_2CH_2CH_2CH_2$O, OOC—$CH_2CH_2CH_2CH_2CH_2$O and OOC—$CH_2CH_2CH_2CH_2CH_2$O in PCL units, respectively, being very similar to the corresponding chemical shifts of PCL in previously reported spectrum [37]. The characteristic signal peak of biotin was very weak because of its low content in the biotin-PEG–PCL_2 copolymer [38].

3.2. Preparation and characterization of micelles

In our previous article, we reported that Y-shaped MPEG–PCL [21] could encapsulate curcumin with higher EE than reported linear MPEG–PCL [39]. So we synthesized a Y-shaped biotion-PG–PCL_2 to study whether if it could show targeting property with high EE. In order to find a suitable formulation for curcumin, we mainly studied effect of length of PCL chain on the EE of curcumin on the basis of fixed PEG chain, temperature and weight ratio of curcumin to copolymer. The results are shown in Table 1. The results indicated that the EE increased from 87.55% to 93.83% with the change of weight ratio of PEG to PCL_2 from 1:1 to 1:1.5. It is well known that PEG and PCL are a hydrophilic and hydrophobic chain, respectively. Linear PCL–PEG–PCL can self-assemble into micelles, in which PEG and PCL are shell and core, respectively [40–42]. So, it was reasonable that biotin-PEG–PCL_2 composed of PEG and PCL could also show the same property. The lipophilic curcumin would be encapsulated into lipophilic PCL core through its molecular interaction with PCL chain. The PEG chain located on the surface of the core strongly interacts with surrounding water to dissolve the drug in water. The longer PCL chain could have stronger affinity with curcumin, resulting in encapsulating a large amount of curcumin into copolymer [43]. So the DL and EE increased with the extension of PCL chain length. However, further extension of PCL chain length could not ulteriorly enhance drug loading. Shown as in Table 1, although the much longer PCL chain could have stronger interaction with curcumin, the EE of curcumin decreased because the water-solubility of biotin-PEG–PCL_{23} was low with further increasing of PCL chain length. In a word, the biotin-PEG–PCL_{22} copolymer was used in the following research based on it higher DL and EE.

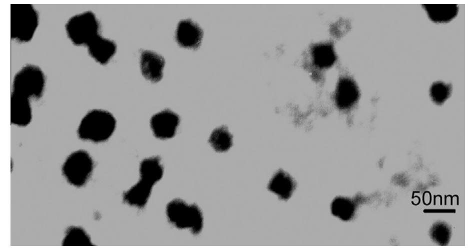

Fig. 2. TEM of curcumin-loaded bio-PEG–PCL_2 micelles (14,000×, the scale bar is 50 nm).

In the study of encapsulating curcumin into the biotin-PEG–PCL_{22} copolymer, the solubility of curcumin in aqueous medium was increased to 1.823 mg/mL, which was 1.704×10^5 times higher than that of free curcumin [44]. This biotin-PEG–PCL_{22} copolymer's CMC was 1×10^{-5} g/mL (S 2), meaning that the copolymeric micelles should show better stability in water. The hemolytic test curve is also shown in S 2. It was found that there was no apparent hemolytic activity in the concentration range of copolymer from 78 to 5000 μg/mL, among which the hemolysis (%) was below 2.34% (<5%) at 576 nm [45].

The zeta potential of curcumin-loaded micelles was 4.62 ± 0.08 mV. The mean particle diameter of curcumin-loaded micelles was 49.28 ± 1.46 nm. The shape of curcumin-loaded biotin-PEG–PCL_2 micelles was discontinuously globular in the TEM photograph provided in Fig. 2.

To prove the existence of drug in micellar curcumin formulation, FT-IR analysis was carried out. FT-IR spectrums of curcumin, curcumin-free and loaded bio-PEG–PCL_2 micelles are listed in S 3. The FT-IR spectrum of blank micelles (S 3B) showed broad bond at 3437 cm^{-1} corresponding to the stretching vibration of hydroxyl. Peaks at 1724 cm^{-1} was attributed to carbonyl's stretching vibration and two peaks at 1196 and 1246 cm^{-1} was due to C—O stretching vibration. Peaks observed at 1108 and 960 cm^{-1} were due to the stretching vibration of PEG chain's C—O, and the double peak at 2945 and 2866 cm^{-1} was due to the C—H stretching vibration. However, the peaks at 3437 and 1724 cm^{-1} shifted to 3416 and 1726 cm^{-1} in micellar formulation of curcumin (S 3A), respectively. At the same time, the phenolic O—H stretching vibration

Table 1
Characteristics of micelles.

Copolymer	Weight ratio of PEG to PCL_2	MW[a]	PDI[a]	EE (%)	DL (%)
Biotin-PEG–PCL_{21}	1:1	5227	1.184	87.55 ± 4.72	11.19 ± 0.58
Biotin-PEG–PCL_{22}	1:1.5	6732	1.104	93.83 ± 0.26	11.94 ± 0.13
Biotin-PEG–PCL_{23}	1:2	8253	1.197	76.63 ± 5.25	9.74 ± 0.64

PDI: Polydispersity index.
[a] It was determined by gel permeation chromatography.

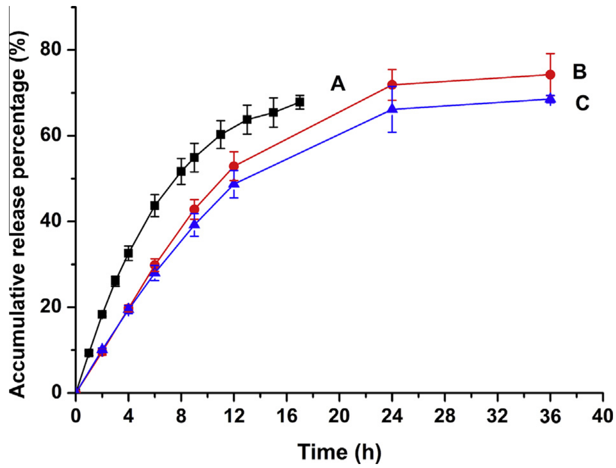

Fig. 3. *In vitro* curcumin release profile from control (A), H_2N–PEG–PCL_2 micelles (B) and biotin-PEG–PCL_2 micelles (C) solution in 40% ethanol saline solution at 37 °C (data are presented as mean ± SD ($n = 3$)).

peak of curcumin (S 3C) at 3510 cm^{-1} also shifted to 3416 cm^{-1} in micellar formulation. These changes confirmed that there was hydrogen bond interaction between drug and copolymer in this formulation [46]. In addition, peaks at 1628 cm^{-1}, 1602 cm^{-1} and 1509 cm^{-1} in curcumin (S 3C) were due to C=C, aryl C=C and C=O/C—C, respectively [47]. The similar peaks at 1623, 1591 and 1515 cm^{-1} in curcumin-loaded micelles (S 3A) were observed, which were absent in blank micelles (S 3), demonstrating that curcumin was dispersed in this formulation [46].

XRD characterization was employed to comprehend the physical state of curcumin in drug-loaded micelles. The characteristic peaks of curcumin appeared in free curcumin (S 3C), demonstrating its crystalline structure [48,49]. Conversely, there were no curcumin's characteristic peaks in drug-loaded micelles (S 3A). This meant that curcumin in micelles was in amorphous, disordered crystalline phase or solid solution state [50–52]. This disordered phase of curcumin in micelles could provide excellent diffusion property through the polymeric framework for the drug molecules, resulting in a sustained release of curcumin from the micelles.

3.3. In vitro release of CUR-NP

The sustained and controlled release of drug from nanoparticulate micelles is a key of always maintaining its effective concentration for prolonged time in body. In addition, the structure difference between biotin-PEG–PCL_2 and H_2N–PEG–PCL_2 might affect the release of curcumin from the corresponding drug-loaded micelles. So, in view of curcumin's low water-solubility and sink condition, the *in vitro* release of curcumin from both of drug-loaded copolymeric micelles was carried out in ethanol–physiological saline solution (40:60 (V/V)). The release curves of curcumin control and curcumin-loaded micelles solution are listed in Fig. 3. It was found that accumulative release percentage of curcumin from control solution achieved >60% at 12 h. During the same time, however, both of drug-loaded micelles only afforded about 50% of curcumin released. The accumulative release percentage of curcumin from drug-loaded biotin-PEG–PCL_2 and H_2N–PEG–PCL_2 micelles was 68.5% and 74.2% at 36 h, respectively. From the results listed in Fig. 3, it was clearly indicated that H_2N–PEG–PCL_2 micelles provided a faster curcumin release than biotin-PEG–PCL_2 micelles in the whole period of release experiment. The possible reason is that the surface of H_2N–PEG–PCL_2 micelles was hydrophilic and easily permeated by water. When these micelles were conjugated with biotin to form biotin-PEG–PCL_2 micelles, surface feature was changed into hydrophobicity and hard to be moist, resulting in the slowing of release [53].

3.4. In vitro cytotoxic test

In this study, cytotoxicities of free curcumin, blank and drug-loaded micelles were investigated in MDA-MB-436 cells by use of MTT method. Study results are shown in Fig. 4. It was found that all blank micelles revealed more than 85% cell viability, showing no obvious toxic effect when they were incubated with MDA-MB-436 cells in 24 and 48 h. All drug-loaded micelles also did not have evident activities when the concentration of curcumin was below 20 μM in 24 h and 48 h [54]. Free curcumin showed 53.88% of cell viability at a concentration of 40 μM in 24 h followed by a decrease to 12.78% in 48 h. It showed a property of concentration-dependent anticancer activities against MDA-MB-436 cells. Its effect was the highest among all drug formulations. The longer

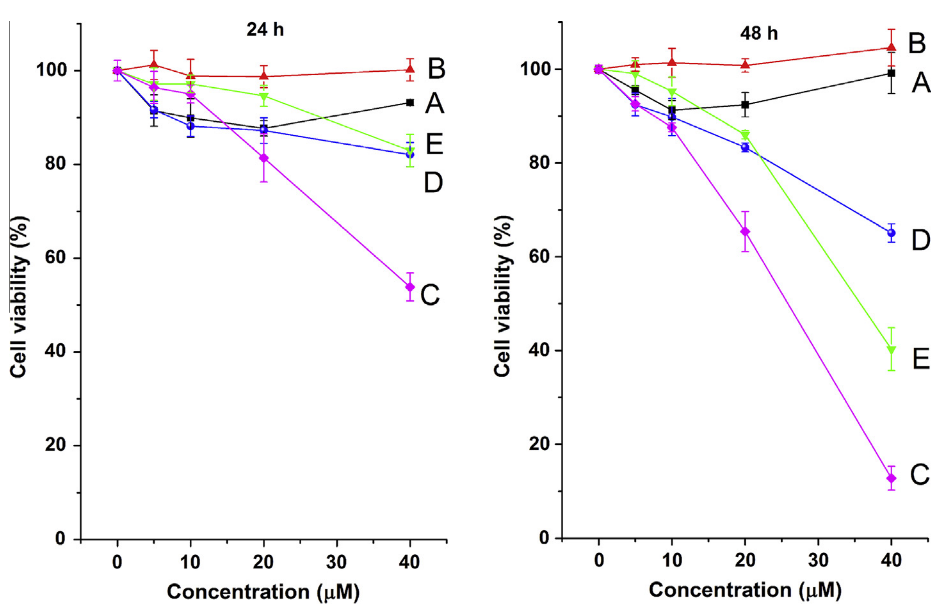

Fig. 4. *In vitro* cytotoxities of blank H_2N–PEG–PCL_2 (A), biotin-PEG–PCL_2 (B), micelles and curcumin in control (C), H_2N–PEG–PCL_2 (D) and biotin-PEG–PCL_2 (E) micelles against MDA-MB-436 cells in 24 h and 48 h (data are presented as mean ± SD ($n = 3$)).

incubation time resulted in the increase of its cytotoxicity. It was interesting that curcumin in biotin-PEG–PCL_2 micelles displayed similar activity with H_2N–PEG–PCL_2 micelles when the concentration of curcumin was at 40 μM in 24 h. However, curcumin in the former micelles became obviously more effective than that in the latter ones at 40 μM when the incubation time was prolonged to 48 h. The survival rate of cancer in the former micellar formulation was only 40%, which was lower than that in H_2N–PEG–PCL_2 micelles.

Biotin is a necessary vitamin whose receptor shows overexpression on the surface of cancer cells quickly proliferating [55]. Just like biotin-PEG–PLA, biotin conjugated with PEG–PCL_2 should locate on the surface of micelles formed by the self-assemble of biotin-PEG–PCL_2 in water [56]. It would be helpful for the recognition and incorporation of receptors to this micelles. Then the uptake of curcumin-loaded biotin-PEG–PCL_2 micelles would be promoted. The confocal fluorescent microscopic photographs about cellular uptake of free curcumin and two curcumin-loaded micelles are shown in Fig. 5. To determine mean fluorescent density in each photograph, ImageJ was used. The mean fluorescent density for free curcumin, curcumin-loaded H_2NPEG–PCL_2 and biotin-PEG–PCL_2 micelles were 0.176, 0.0161 and 0.0645. So the fluorescent intensity of curcumin-loaded biotin-PEG–PCL_2 micelles in cancer cells was higher than that of curcumin-loaded H_2N-PEG–PCL_2 micelles, showing that cellular uptake of the former was improved by introduction of biotin into the copolymeric micelles. The uptake of free curcumin was obviously higher than that of drug-loaded micelles, which might be originated from the difference between endocytosis of the nanocarrier and the diffusion of a small-molecule drug [57,58].

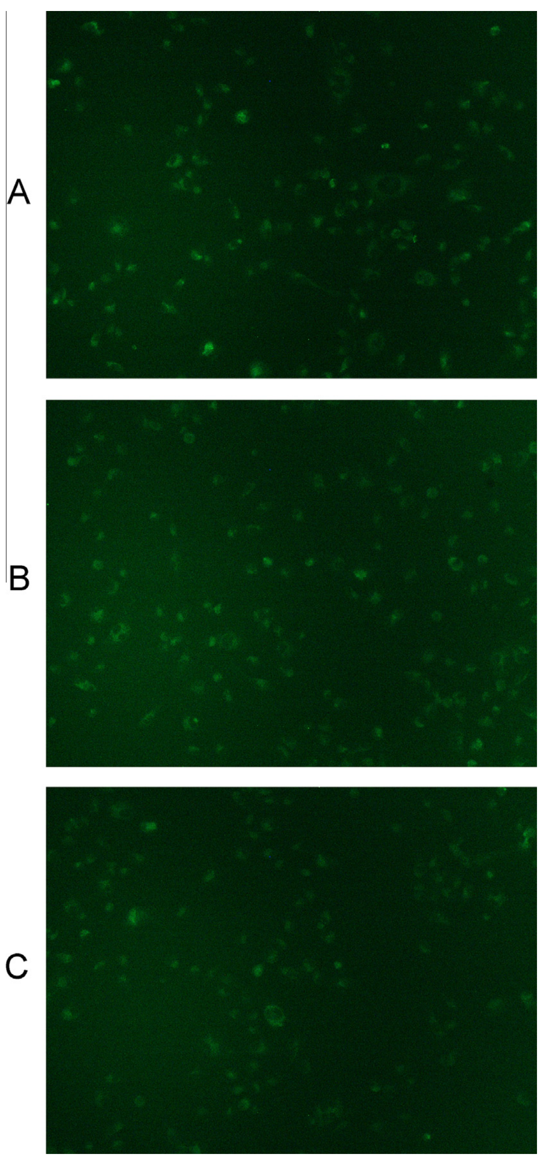

Fig. 5. *In vitro* uptake of curcumin in control (A), biotin-PEG–PCL_2 (B) and H_2N–PEG–PCL_2 (C) micelles in MDA-MB-436 cells in 2 h.

For the sake of killing cancer cells, it is important that curcumin in drug-loaded micelles in cells must be firstly released to afford a minimum valid concentration. The uptake of curcumin-loaded micelles continuously proceeds during the whole test, but the release of curcumin might be a decisive factor when the concentration of curcumin was in the range of 0–20 μM. The *in vitro* release test results in Section 3.4 demonstrated that curcumin's release from drug-loaded micelles was slower than that from free curcumin control solution, and its release from biotin-PEG–PCL_2 micelles was the lowest among the three formulations. This would lead to a lower accumulation of curcumin in cancer cells during the same time. As a result, in the range of 0–20 μM, controlled release made cytotoxicity of curcumin in micelles very low in 24 h and 48 h.

However, the cancer cell selective uptake of curcumin-loaded biotin-PEG–PCL_2 micelles was obviously enhanced, surpassing curcumin's release via the induction of biotin when the concentration of curcumin achieved 40 μM. Although the release rate of curcumin from biotin-PEG–PCL_2 micelles was always slower than that from H_2N–PEG–PCL_2 micelles, uptake of the former was much higher than the latter. This change induced by biotin greatly enhanced curcumin's concentration in MDA-MB-436 cells, and the release amount of curcumin from micelles without biotin only mildly enhanced at the same time. As a result, amounts of curcumin released in MDA-MB-436 cells from the two drug-loaded micelles were analogous in 24 h when the curcumin's concentration was set at 40 μM, showing similar cytotoxicities against MDA-MB-436 cells. When the incubation time was extended to 48 h, more and more curcumin in the biotin-PEG–PCL_2 micelles were released into cancer cells, resulting in higher cytotoxic effect than that of the latter. In conclusion, the biotin molecular could improve the cancer cell uptake of drug-loaded micelles, showing better property of cancer cells target at higher dosage of drug for a long incubation time.

4. Conclusion

Biotin-PEG–PCL_2 obtained from O-alkylation, alkaline ring-opening, radical addition, N-acylation and ring-opening polymerization with APEG as raw material could successfully encapsulate curcumin with higher encapsulating efficiency. It obviously enhanced curcumin's water-solubility and controlled curcumin's release which was slower than H_2N–PEG–PCL_2 micelles. To evaluate biotin's targeting ability to cancer cell, we studied *in vitro* cytotoxicities of curcumin-loaded biotin-PEG–PCL_2 and H_2N–PEG–PCL_2 micelles against MDA-MB-436 cells. The research results indicated that biotin could promote the uptake of curcumin-loaded micelles, showing better anticancer activity for a long incubation time. In a word, the use of Y-type biotin-PEG–PCL_2 polymeric micelles as a novel drug delivery system should be feasible.

Disclosures

The authors report no conflicts of interest in this work.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcis.2014.11.073.

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